Review Article

Finding a Place for New Approach Methodologies (NAMs) in Biomedical Research

Alexandra Schaffert[1], Arti Ahluwalia[2], Jeffrey Bajramovic[3], Nuno H. Franco[4], Winfried Neuhaus[5], I. Anna S. Olsson[4], Ioanna Sandvig[6]

1. Tampere Institute for Advanced Study, Tampere University, Tampere, Finland; 2. Centro 3R, Department of Information Engineering, University of Pisa, Italy; 3. 3Rs Centre Utrecht, Utrecht University, Netherlands; 4. i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal; 5. AIT Austrian Institute of Technology, Vienna, Austria; 6. Department of Neuromedicine and Movement Science, Norwegian University of Science and Technology, Norway

New Approach Methodologies (NAMs) can greatly contribute to the Replacement and Reduction of animal experiments in biomedical science. However, while NAMs have seen considerable investment and adoption in toxicology, this growth has not found a parallel in basic and translational biomedical research. Here, we examine the opportunities and the challenges of systematic implementation of NAMs in biomedical research.

We demonstrate that NAMs offer significant scientific value by enabling tailored investigations into biological mechanisms and disease processes, enhancing human relevance, and harnessing cutting-edge technological innovations. We highlight key areas—neuroscience, cardiovascular research, and oncology—where NAMs have a distinct advantage and the potential to drive major advancements. However, realizing their full potential requires overcoming critical barriers, including technological limitations, gaps in education and training, and insufficient funding. To facilitate broader adoption of NAMs in biomedical research, we propose the use of Clinical Outcome Pathways (COPs) as a guiding framework. COPs provide a structured approach to mapping knowledge of biological events from molecular initiating events to clinical outcomes, integrating diverse NAM-generated data into experimental workflows. Finally, we outline a stepwise strategy to accelerate the adoption of NAMs, emphasizing interdisciplinary collaboration, parallel studies alongside existing animal models, and sustained investment in infrastructure and education.

 doi.org/10.32388/L13YBA

Corresponding author: Alexandra Schaffert, alexandra.schaffert@tuni.fi

1. Introduction

The adoption of New Approach Methodologies (NAMs) in biomedical research is receiving increasing attention, driven by advancements in technology and the political discussion concerning animal testing [1]. Originally, the term New Approach Methodology was coined in the context of regulatory toxicology [2] with the specific goal of replacing a conventional toxicological animal experiment with an "alternative approach." Since then, NAMs have become a central focus in toxicology, supported by regulatory mandates, dedicated funding initiatives, and standardized methodological frameworks [2][3]. Toxicological regulatory frameworks continue to evolve, with ongoing efforts to define where and how NAMs can effectively reduce and replace animal models. The recent development of a comprehensive EU Commission roadmap for phasing out animal testing in chemical safety assessment further highlights this paradigm shift and provides concrete recommendations to accelerate the transition toward a non-animal regulatory framework [4]. While these efforts collectively underscore a shared commitment and a significant shift toward reducing and replacing animal testing with more ethical and scientifically advanced alternatives in toxicology, the expansion of NAMs into the broader field of biomedical research has not paralleled this growth. This disparity warrants attention, given the scale of animal use in biomedical research. According to a 2019 report on the use of animals for scientific purposes in EU Member States, approximately 70% of animals were used in basic, applied, and translational biomedical research [5], underscoring the influence that the implementation of NAMs in these fields could have on overall animal use numbers.

As the name suggests, in the broadest sense, NAMs are methodologies based on new approaches and paradigms that do not require interspecies extrapolation.

Thus, if applied to human biology, NAMs are ideally animal-free, both in concept as well as in their downstream workflow. NAMs do not necessarily provide a one-to-one substitute for conventional animal-based techniques but rather offer novel ways to address research questions that animal models might not be able to answer. As such, they can act as specific replacements (surrogates for existing animal tests) or as proactive replacements, opening new and potentially unprecedented avenues of research without relying on animals[6].

Full definition can be found in Ahluwalia et al. 2025 (submitted to Lab Animal)

What is a New Approach Methodology (NAM)?

The disparity between NAM adoption in toxicology versus biomedicine may partly reflect differences in how models are used, in the overall goals, or in the research community dynamics of the two fields. In scientific terms, biomedical research is distinct from toxicology in a way that presents unique challenges and opportunities for the integration of NAMs. Unlike the rather pragmatic toxicological studies, which focus primarily on risk assessment, the objective of biomedical research is to understand complex disease mechanisms, develop therapeutic strategies, evaluate treatment efficacy, and identify biomarkers. Meeting these objectives requires diverse methodologies that can mimic the intricate biological interactions occurring in living organisms. A successful introduction of NAMs depends on understanding to what extent different models are actually helpful in providing answers to relevant research questions.

Biomedical research appears to be at a critical inflection point, as discussions intensify about the lack of reproducibility and the limited predictive value of animal models for human biology [7]. In parallel, biomedical researchers must navigate the challenges, including peer resistance and methods of validation, and opportunities, such as innovation and interdisciplinarity, of adopting NAMs more broadly.

Given the significant strides made with NAMs in toxicology and their ethical advantages, it is pertinent to ask whether and to what extent NAMs can be as effectively implemented in the more complex area of biomedical research, or in other words: What is the place for NAMs in biomedical research?

Hence, we explore (a) whether and how NAMs can add value, (b) what the biggest challenges to increasing NAM implementation are, and (c) what we can learn from toxicology. Moreover, we propose Clinical Outcome Pathways (COPs) as a conceptual framework to guide the use of NAMs in biomedical research questions and lay out a stepwise approach to broaden their adoption.

2. What added scientific value can NAMs offer in biomedical research?

The implementation of NAMs provides a unique opportunity to advance biomedical research by generating species-specific, directly relevant data that are often inaccessible through traditional animal models. Rather than simply serving as substitutes for animal experiments, NAMs embody a paradigm shift in how scientific questions can be approached and answered, as highlighted by recent efforts to clarify their definition and scope. To broaden the adoption of NAMs, it is essential to move beyond the question of regulatory obligation, "Do I have to use animal models?", and instead focus on the scientific possibilities: "How can NAMs enhance and transform my research?" This section examines the distinctive scientific value that NAMs can bring to biomedical research, pushing its boundaries beyond what is currently achievable.

2.1. Ethical Considerations

The concept of ethical animal use is often framed by the "3Rs": **Replacement** (seeking non-animal approaches whenever scientifically adequate), **Reduction** (obtaining the required information from fewer animals), and **Refinement** (minimizing pain or distress when animals are still needed) [6]. NAMs circumvent the need for cross-species validation by using models based on human cells or data. Consequently, NAMs renew attention to the first R—Replacement—by making alternative approaches technically viable on a scale unimaginable in 1959, when Russell and Burch originally framed the 3Rs as a hierarchy beginning with that very question [8]. In cases where complete replacement is not yet possible, NAM-based high-content screens or computational filters can eliminate non-viable hypotheses and focus any subsequent *in vivo* work on the most informative questions, substantially lowering total animal use.

While NAMs mitigate many ethical concerns, they are not entirely without ethical considerations of their own [9]. The use of human biological material, such as tissues or stem cells, raises questions about donor

consent and the equitable sourcing of materials. Additionally, many *in vitro* workflows cannot be described as complete replacements, since they rely on animal-derived products such as fetal bovine serum and basement membrane matrices (like Matrigel). However, recent advances are driving the development of chemically defined, xeno-free culture media that replace FBS with recombinant growth factors, hydrogel-based extracellular matrices derived from synthetic polymers or plant-based sources, and serum-free differentiation protocols for stem cells [10][11]. These innovations not only enhance the ethical acceptability of NAMs but also improve reproducibility and reduce batch-to-batch variability associated with biologically derived components.

2.2. Direct Human Relevance

One of the primary limitations of animal models is the difficulty of extrapolating results to humans due to species-specific differences in physiology, pathology, and drug responses. Additionally, animal models are typically bred or genetically modified to have uniform characteristics, such as the same genetic background, age, sex, or health status, to minimize variability within experiments. This standardization ensures consistency in experimental results but limits the ability of these models to reflect the genetic, biological, and environmental diversity seen in human populations.

NAMs, as species-specific methodologies, address these issues by leveraging human-derived cells, tissues, and data to investigate biological pathways and disease mechanisms directly relevant to humans. These models can include cells from diverse genetic and demographic backgrounds, allowing for the study of variability in disease mechanisms and treatment responses. For example, patient-derived organoids can represent specific genetic mutations, ethnicities, or environmental exposures, offering a more accurate reflection of human diversity compared to standardized animal models [12].

Although NAMs can substantially enhance human relevance, their current limitations must be acknowledged. Organoids and *in vitro* systems generally lack the full complexity of integrated human physiology, such as immune system interactions, vascularization, and multi-organ crosstalk. Importantly, however, most NAMs are not intended to replicate the entire organism but rather to model well-defined aspects of human biology with precision and control. For instance, human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes in 3D tissue constructs can be used to investigate cardiac electrophysiology or drug-induced arrhythmias, processes that are difficult to extrapolate from animal models due to fundamental species differences in cardiac ion channel expression and function [13]. A number of efforts are underway to model more systemic and integrated aspects of human

biology using species-specific approaches. Notably, organ-on-chip technologies have seen rapid progress in recent years. By incorporating microfluidic channels to simulate blood flow and tissue-tissue interfaces, these platforms enable the investigation of dynamic physiological processes, such as immune cell trafficking, vascular responses, and real-time interactions between organ systems. For example, lung-on-a-chip and heart-lung chip models have been used to study how respiratory and cardiovascular tissues interact in the context of disease or drug exposure [14]. In parallel, advances in microphysiological systems allow the functional linking of multiple human organ models, including liver, gut, and kidney, supporting more accurate studies of pharmacokinetics, metabolism, and organ-organ crosstalk in a human-relevant setting [15]. Further notable developments include the Virtual Physiological Human (VPH) initiative, which integrates computational and experimental data to build whole-body models, and the creation of endocrine system-on-a-chip platforms to study hormone-mediated inter-organ communication [16][17].

Currently, no single NAM can fully replicate whole-body complexity, but each can be tuned to dissect a defined human mechanism or pathway and thereby accelerate the translation of findings into clinical applications. Designing and validating these models against clinical benchmarks, i.e., the physiological conditions, functional parameters, and outcome measures observed in patients, ensures that NAM data inform directly about human disease and therapy. When such experimentally anchored NAM data are combined with *in silico* models and clinical datasets, gaps between isolated *in vitro* findings and whole-body physiology can be reduced.

2.3. Precision and Control

Given that animals and humans are inherently complex and not entirely understood systems, some researchers argue that it may be scientifically flawed to assume that findings in one species can reliably predict outcomes in another, regardless of how rigorous the experimental standards are [18]. Confounding factors, interactions between systems (e.g., immune, nervous, and endocrine systems), and compensatory mechanisms can not only lead to unexpected or unpredictable outcomes but also obscure the effects of targeted interventions, making it difficult to connect observed outcomes to their underlying causes.

NAMs, defined as species-specific approaches that generate data directly relevant to the target organism, offer a solution to these challenges. Variables such as the cellular environment, genetic background, and specific signaling pathways can be controlled, enabling focused investigations of particular mechanisms. For instance, cell cultures, engineered tissues, and organ systems allow researchers to isolate and analyze

a drug's direct effects on specific cell types or tissues, without the systemic complexities of whole organisms. While this approach provides detailed mechanistic insights, for the time being, complementary models are required to assess pharmacokinetics, distribution, and overall efficacy in a physiological context.

Importantly, NAMs also offer unique advantages for capturing dynamic processes across spatiotemporal scales. *In vitro* models can be designed to mimic physiological conditions, furnishing insights into spatial and real-time interactions that are difficult or impossible to observe *in vivo*. For instance, cells can be cultured on biomaterials with spatial stiffness gradients to study microenvironment-driven motility, differentiation, or drug response. Technologies exploiting microfluidic devices and real-time imaging allow the investigation of time- and flow-dependent phenomena. These approaches enable researchers to analyze the progression of biological events, such as drug-target interactions or disease mechanisms, in controlled and reproducible settings [19][20]. Additionally, emerging machine intelligence-based *in silico* models can incorporate patient-specific data, providing insights that are directly relevant to human physiology and reducing reliance on animal models [21].

Yet, chronic, months-to-years studies remain a challenge for most *in vitro* systems; only recently have long-lived organoids and perfused bioreactor cultures begun to extend observation windows toward modeling aging or late-stage pathology. Thus, while NAMs excel at resolving fast or mid-term dynamics, complementary strategies are still needed to emulate very long-term disease trajectories.

2.4. Technological Innovation

NAMs leverage cutting-edge technologies such as organ-on-chip systems, microphysiological platforms, omics applications, imaging, biomaterials, nanotechnology, and artificial intelligence, enabling observations and experimental designs that were not feasible just a decade ago. These tools have broadened the scope of biomedical research, making it possible to address new types of questions with improved precision and direct human relevance.

Besides microphysiological organ-on-chip-type systems and organoids, a vast array of methods and tools are now available for live imaging at high resolution and depth of penetration. Advances in super-resolution microscopy technologies coupled with novel fluorophores allow interrogating single RNA molecules in cells [22], while high-depth imaging methods such as photoacoustic and light-sheet microscopy can allow functional acquisition [23][24].

In parallel, omics technologies, such as single-cell transcriptomics and proteomics, have advanced to the point where they can provide highly detailed snapshots of cellular processes. For instance, these techniques are now being integrated into organoid research, allowing researchers to monitor how individual cells within a tumor organoid respond to therapies at the molecular level [25][26]. This unprecedented level of resolution has the potential to drive breakthroughs in personalized medicine.

Artificial intelligence and machine learning are playing an increasingly central role in interpreting the large, complex datasets produced by modern NAMs. In recent years, machine learning models have facilitated the prediction of drug efficacy and safety based on human-relevant transcriptomic and proteomic data, as well as the modeling of adverse drug reactions [27].

Looking forward, technological innovation in NAMs is expected to continue at a rapid pace. Advances in biofabrication, such as 3D bioprinting, may allow for the creation of even more complex tissue constructs, including vascularized and innervated organ models. Machine learning models are likely to become more predictive as they integrate increasingly diverse datasets, potentially enabling patient- or population-specific simulations of disease progression or therapeutic outcomes. These developments will expand the range of questions that can be addressed using NAMs and further reduce the need for animal models in biomedical research.

3. Application of NAMs in the biomedical field

Neuroscience, cardiovascular research, and cancer research are the focus areas of the IMPROVE EU COST action [28]. Thus, we have selected key areas within these disciplines that hold potential for significant impact in the field (Table 1). Here, we discuss only a small selection of available NAMs in these areas; however, in 2022, the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) conducted a large report on available NAMs in biomedical research areas such as neurodegenerative disorders, cardiovascular diseases, and different topics of cancer research [5].

Neuroscience	Cardiovascular research	Cancer research
Disease Modeling and Drug Discovery: NAMs offer unprecedented opportunities to model complex neurological diseases by mimicking human-specific disease processes and genetic variations, enabling the discovery of novel treatments more relevant to humans than those identified using animal models. Recent examples include human midbrain organoids that reproduce dopaminergic-neuron loss and α-synuclein pathology, providing a platform for Parkinson's disease drug screening [29], and high-throughput screening of C9ORF72-mutant iPSC motor neurons that identified spliceosome modulators mitigating toxic RNA foci in amyotrophic lateral sclerosis [30]. However, widely applied organoid models currently face significant challenges, such as long-term viability and aging, which are critical for modeling neurodegenerative diseases like Alzheimer's, where age is a key predisposing factor. Efforts are underway to improve these models, but it is important to acknowledge that organoids are not yet a viable replacement for several animal	**Disease Modeling and Drug Discovery:** Species differences frequently limit the translation of findings from rodents to humans due to fundamental differences in heart rates, blood pressure, repolarizing currents, and action-potential morphology, contributing significantly to drug withdrawals [32]. Complex NAMs using human cell lines, such as heart-on-chip systems, bridge this gap. They overcome the limitations of 2D cultures by providing highly biomimetic microenvironments with 3D cell culture technology. These platforms can simulate cardiomyocyte beating and electrical conduction in a human-relevant context, and the integration of biosensors allows real-time, multiplex functional readouts, although generally at a lower throughput than plate-based assays. For instance, hiPSC-derived cardiomyocyte models on heart-on-chip platforms enable the rapid assessment of changes in beating patterns and ion channel function and can be more sensitive to human-relevant ion-channel	**Disease Modeling and Drug Discovery:** High-throughput screening (HTS) using human cell-based assays, combined with computational drug discovery tools, offers significant advantages in terms of time, cost, and resource efficiency by allowing the simultaneous testing of thousands of compounds, reducing the labor-intensive and expensive nature of traditional drug discovery approaches. Microfluidic chip organ models show promise in facilitating the rapid and cost-effective identification of effective drug combinations and advancing personalized treatment strategies [36]. A recent study conducted the largest high-throughput drug screening on patient-derived low-grade serous carcinoma (LGSOC) cell lines, identifying 60 high-confidence compounds and providing new therapeutic avenues for this chemo-resistant ovarian cancer subtype [37]. Nonetheless, most HTS assays still rely on 2D cultures that lack stromal, immune, and vascular complexity, limiting mechanistic insight. Research addressing this gap is

Neuroscience	Cardiovascular research	Cancer research
models of central nervous system diseases. On the other hand, advanced 2D or 3D neural network models capture the intrinsic self-organizing principles of neurons in the brain very effectively [31].	liabilities (e.g., hERG/QT) [33][34]. Data-driven models (including neural networks) trained on *in vitro* and clinical safety data improve the prediction of kinase-inhibitor cardiotoxicity and can aid preclinical prioritization [35].	underway, e.g., tumor-microenvironment-on-a-chip platforms integrating multiple compartments, yet they remain labor-intensive, low-throughput, and only partly validated against clinical outcomes [38].
Organ development: The intrinsic self-organizing activity of neurons *in vitro* enables the formation of neural networks with structural and functional complexity, and network dynamics closely mirroring those of the brain, allowing for selective manipulation under physiological and pathological conditions. Recent advances include brain organoids and assembloids that recapitulate aspects of human neurodevelopment, region-specific patterning, and migration of different neuronal subtypes. Multicellular models allow the investigation of neuron-glia interactions and neuroinflammatory mechanisms [39]. The *in vitro* battery developed by the PARC consortium to replace animal studies for rapid, robust screens of developmental neurotoxicity could also be highly informative for basic neurobiology [40]. Conventional transwell blood-brain-barrier co-cultures and newer microfluidic	**Organ development:** Recent advances in human *in vitro* cardiac models, such as multi-chamber cardioids [43], patterned heart tube organoids [44], blood-generating cardiac organoids [45], vascularized cardiac organoids [46], and multi-cell type engineered heart tissues [47], enable the recapitulation of key processes in human heart development, including chamber formation, patterning, vascularization, and functional maturation. These next-generation platforms have proven valuable for investigating the mechanisms underlying congenital heart defects, maternal and drug-induced teratogenicity, and complex cell-cell interactions, providing mechanistic and translational insights that are not accessible in animal models. Moreover, human-specific aging features can be modeled by adjusting scaffold stiffness and the extracellular microenvironment in	**Precision medicine:** Patient-derived organoids (PDOs) replicate tumor heterogeneity and molecular profiles, providing a non-animal alternative for selecting cancer therapies. For example, breast cancer organoids from biopsies retain HER2, ER, and PR markers for targeted drug testing [49]. Similarly, PDOs from various organs have already been generated [50]. These models mirror the genetic and phenotypic diversity of tumors and capture individual responses to therapy. Gene-editing tools enhance organoid models by enabling precise mutation studies; e.g., CRISPR-modified colon cancer spheroids have been used to investigate therapeutic targets related to stemness and therapy resistance mechanisms [51]. Integration with multi-omics profiling, like single-cell transcriptomics, allows deeper exploration of tumor heterogeneity

Neuroscience	Cardiovascular research	Cancer research
chip-based platforms reproduce selective permeability and vascular-neural signaling with assay formats ranging from high-throughput screening to mechanistic interrogation [41][42].	3D tissues, addressing age-related cardiac changes that are difficult to model in short-lived rodents [48].	and identification of novel therapeutic targets for subpopulations within a tumor [25] [26].
Disease mechanisms: Integration with technologies such as microfluidic devices, electrophysiological platforms such as microelectrode arrays (MEAs), and microfluidic MEAs offers a wide range of opportunities to structure, selectively manipulate, and study hierarchical neural networks and complex microcircuits to understand function or dysfunction in the presence of inherent or induced pathological perturbations. For example, microfluidic MEA systems enable the precise organization of neural cultures and targeted delivery of compounds for controlled analysis of circuit function [52]. Similarly, combining high-density MEAs with microfluidic patterning creates modular neuronal networks with enhanced complexity and synchronization [53]. This allows dynamic studies at subcellular, synaptic, and network levels, advancing our understanding of neural configurations on	**Disease mechanisms:** Human iPSC-derived 3D cardiac microtissues reveal mutation-specific long-/short-QT phenotypes that rodents often miss because of species-specific repolarization and action-potential morphology [55]. Heart-on-chip platforms show great advancements in recapitulating diseases *in vitro*, allowing controlled flow and oxygen and electrical gradients; e.g., a myocardial infarct border-zone-on-a-chip with controlled O_2 gradients revealed region-dependent impairments in calcium handling, stress generation, and inflammatory signaling during simulated ischemia-reoxygenation[56]. Atherosclerosis-on-chip devices under physiological shear allow modeling of LDL loading, endothelial activation, and monocyte influx in real time, mapping plaque evolution step by step [57]. In addition, combined computational fluid dynamics models and agent-based models	**Disease mechanisms:** The tumor microenvironment (TME) is central to cancer progression, metastasis, and treatment resistance, yet its complexity makes it challenging to model. Recent advances in PDO systems allow key TME features to be recreated, such as co-culture with immune cells, cancer-associated fibroblasts, and extracellular matrix components. For example, co-culturing PDOs with tumor-infiltrating lymphocytes (TILs) has revealed new mechanisms of TIL activation, degranulation, and migration [59]. Microfluidic organ-on-chip systems further simulate dynamic interactions between tumor, immune, and vascular components, allowing studies of immune suppression and epithelial-endothelial interactions. While replicating vascularization and hypoxia remains difficult, bioengineered hydrogels and advanced chips are constantly

Neuroscience	Cardiovascular research	Cancer research
spatiotemporal scales difficult to achieve *in vivo*. When combined with advanced computational models, including AI-based models, these network models provide unique insights into neural dynamics under healthy and perturbed conditions [54].	reveal how low shear, LDL influx, and smooth-muscle dynamics drive plaque initiation and destabilization [58]. However, although some chips now run for weeks and cardiac organoids approach adult-like maturation, full maturity, months-long stability, and systemic neurohormonal cues remain outstanding challenges.	improving TME modeling [50]. Despite progress, most vascular networks remain immature with limited length-scale perfusion, and few platforms yet integrate the full stromal and immune diversity required for systemic cues and long-term evolution.

Table 1. Applications in the biomedical field and key areas where NAMs can have a unique advantage leading to significant improvements

4. Challenges for the implementation of NAMs in biomedical research

Integrating NAMs into biomedical research presents significant challenges, particularly for researchers accustomed primarily to animal models. Animal models have been the cornerstone of biomedical research for decades, making the adoption of NAMs both scientifically and institutionally complex.

Scientific and Technical Limitations: One of the primary technical limitations is that NAMs, by design, focus on selected components or pathways within the target species, making it difficult to capture the complexity of whole-organism interactions such as integrated metabolism, immune responses, and pharmacokinetics. While advanced organoid systems and organ-on-chip technologies have improved the modeling of tissue-specific and some multi-system processes, these platforms often lack features such as full vascularization, adult-like tissue maturity, and comprehensive immune system integration. For instance, many organoids are derived from embryonic or stem cell sources, making them more representative of immature tissues rather than the adult phenotypes seen in most disease contexts.

Emerging tools, such as organ-on-chip platforms that incorporate immune components [60] or co-culture systems simulating tumor-immune interactions [38], show promise in addressing these gaps at

least partly. Moreover, the use of machine learning methods that can handle large amounts of data to identify patterns and principal components to help construct better models should be better exploited. Nonetheless, state-of-the-art NAM technologies can certainly help broaden the horizons of biomedical research by complementing and supporting *in vivo* methods.

Assessing NAMs Capabilities and Constraints: Just as certain animal models have limitations for specific applications, different NAMs are better suited to particular research needs based on their unique capabilities and constraints. While animal models often allow for the direct observation of disease manifestations, NAMs typically produce data that focus on underlying mechanisms or functional parameters, such as protein functionality or cellular processes. This functional focus can make their predictive value less immediately apparent. A poor understanding of NAMs' relevance, especially in the absence of clear validation data linking the model to clinical outcomes, can hinder their implementation. Because new NAMs often require model characterization and iterative optimization, their adoption can be slow, especially when researchers are uncertain about how well a given NAM addresses their specific scientific question. This creates a vicious cycle: Limited use means fewer opportunities to generate the comparative data needed to establish confidence in NAMs, which in turn slows further uptake. Breaking this cycle requires focused efforts in implementation, benchmarking, and transparent characterization of the performance of NAMs, fostering confidence in these methods across the research community.

Training and Education: Limited exposure to NAMs during formal education or training contributes to a lack of awareness of these methods. There is a significant need for education and training in NAMs, including an understanding of their capabilities, limitations, and appropriate applications. This gap is especially pronounced for *in silico* approaches, which require specialized computational skills not traditionally emphasized in biomedical curricula. Integrating comprehensive NAMs education, including practical experience, mechanistic understanding, and critical evaluation, into undergraduate, graduate, and professional development programs is essential for fostering innovation and enabling informed uptake across the biomedical sciences.

Economic and Institutional Barriers: Implementing NAMs can require a significant upfront investment in new technologies, equipment, and training. Research institutions and funding bodies may be hesitant to allocate resources toward these methods without clear scientific, regulatory, or economic incentives. Additionally, institutional rigidity and the 'this is how it's always been done' mindset can slow down the adoption of innovative approaches. Overcoming these obstacles will require targeted incentives,

institutional leadership, and recognition of the long-term scientific and societal value offered by species-specific research models.

Funding Limitations: The development and adoption of NAMs require significant financial and infrastructural resources. Research funding is typically allocated to projects addressing disease mechanisms or basic biological processes, which might favor established methods like animal models. While this approach ensures feasibility, it limits opportunities to develop and adopt NAMs. Directed funding for NAM development, as seen in toxicology, is critical for advancing biomedical research. The success of the recent Horizon Europe's call for non-animal and human-based tools and strategies for biomedical research demonstrates the research community's eagerness to engage in NAM innovation when adequate resources are available [61].

Perception Challenges: Implementing NAMs requires specialized expertise and protocol customization, contrary to the misconception that their adoption and use are straightforward. Rapidly evolving technologies further necessitate continuous updates and adaptations. Collaboration and knowledge sharing among NAM practitioners are essential to address these challenges. Detailed protocols, hands-on workshops, and comprehensive training resources can help researchers navigate these complexities and build confidence in using NAMs effectively.

5. Accelerating the integration of NAMs into biomedical research: Lessons from toxicology

The field of toxicology has demonstrated that focused efforts in technology development, regulatory implementation, policy support, and collaboration can shift an entire discipline toward non-animal alternatives. Biomedical research, despite its broader scope and complexity, can draw important lessons from toxicology's experience to address the challenges of NAM integration. Here, we discuss frameworks and strategies that have facilitated NAM adoption in toxicology and consider how these can be adapted to accelerate their use in translational biomedical research.

The concept of Clinical Outcome Pathways supports NAM implementation

Promoting the implementation of NAMs in biomedical research requires a change in how research designs are conceived and applied. While *in vitro* and *in silico* approaches, which are naturally aligned with the 3Rs, have been used for decades, the concept of NAMs extends this framework by emphasizing the

development of human-specific methodologies that supersede animal use. To achieve this, a systematic effort is needed to identify and implement NAMs at different stages of biomedical research, ensuring their alignment with the goals of the 3Rs while advancing scientific standards.

In this regard, the Adverse Outcome Pathway (AOP) framework has been instrumental in achieving NAM implementation in toxicological research and regulatory contexts, and similarly, AOPs could promote the systematic implementation of NAMs in biomedical research. AOPs offer a structured, transparent way to organize existing knowledge and experimental evidence by linking a Molecular Initiating Event (MIE) through a cascade of Key Events (KEs) at multiple biological levels with a final Adverse Outcome (AO). Thus, multiple streams of data (*in silico*, *in vitro*, *in vivo*, epidemiological, clinical, etc.) are integrated into a single AOP. This not only helps to assess the weight of evidence and identify knowledge gaps but also guides the targeted development of NAMs to address the specific KEs and integrate NAM data into a coherent biological context. Using AOPs as a guide, the toxicology community has learned to reframe its research questions from relying on apical endpoints observed *in vivo* to focusing on investigating the underlying mechanisms and implementing integrated testing strategies based entirely on NAMs for regulatory chemical safety assessments [62].

In a similar way, concepts like the Clinical Outcome Pathway (COP) framework offer an opportunity analogous to the role of the AOP framework. While AOPs in toxicology focus on adverse effects caused by chemical exposure, COPs describe biological processes leading to desired clinical outcomes reaching across different biological levels, spanning molecular to organism [63]. Like AOPs, COPs are composed of three main components (Figure 1): the Molecular Initiating Events (MIE), Intermediate Events (IE) on multiple biological levels, and Clinical Outcomes (CO). Korn, Thieme, Alves, Yeakey, Borba, Capuzzi, Fecho, Bizon, Edwards, Chirkova, Colvis, Southall, Austin, Muratov and Tropsha [63] describe COPs as drug-induced pathways, which result in improvement of a disease-related outcome, but generally they could describe any biological pathway of interest, with MIEs being induced by endogenous or exogenous ligands or stimuli. Leaning on this approach, Heesbeen, Bijlsma, Risseeuw, Hessel and Groenink [64] applied the AOP concept to map the sequence of biological events from TNF-α receptor activation (as the MIE) to impairment of fear learning. While highlighting key checkpoints such as glutamatergic and serotonergic signaling and neuronal cell death, this approach identified knowledge gaps, such as the time-dependent effects of TNF-α, demonstrating how such concepts can structure multi-level biological data to uncover mechanistic insights.

Similar to the AOP framework, single COPs are not isolated entities but can be interconnected by shared intermediate events to form larger, integrated networks that reflect the complexity of biological systems. This interconnected nature of COPs also enhances their utility in addressing complex diseases involving multiple systems or tissues. An example can be seen in the CIAO project (https://www.ciao-covid.net/), which applied the AOP framework to comprehensively map COVID-19 pathogenesis, resulting in the development of 24 individual AOPs, which were consolidated into a network of 17 AOPs ranked by quality [65]. By visualizing how these pathways share key events such as hyperinflammation and coagulation, the CIAO initiative highlighted critical mechanistic hubs, uncovered tissue-specific processes, and identified knowledge gaps that can direct future research. Similarly, COPs could be used to create networks for diseases like diabetes, where pathways describing insulin resistance in muscle, liver, and adipose tissue are mechanistically linked to systemic outcomes such as hyperglycemia and vascular complications. This effort can guide where to build new NAMs, ensure they focus on the most relevant mechanisms, and show how NAM data can be interpreted in the context of broader disease biology. In this way, COPs actively support the advancement of NAMs by guiding both the development of human-specific models and the interpretation of their results within a broader understanding of disease processes.

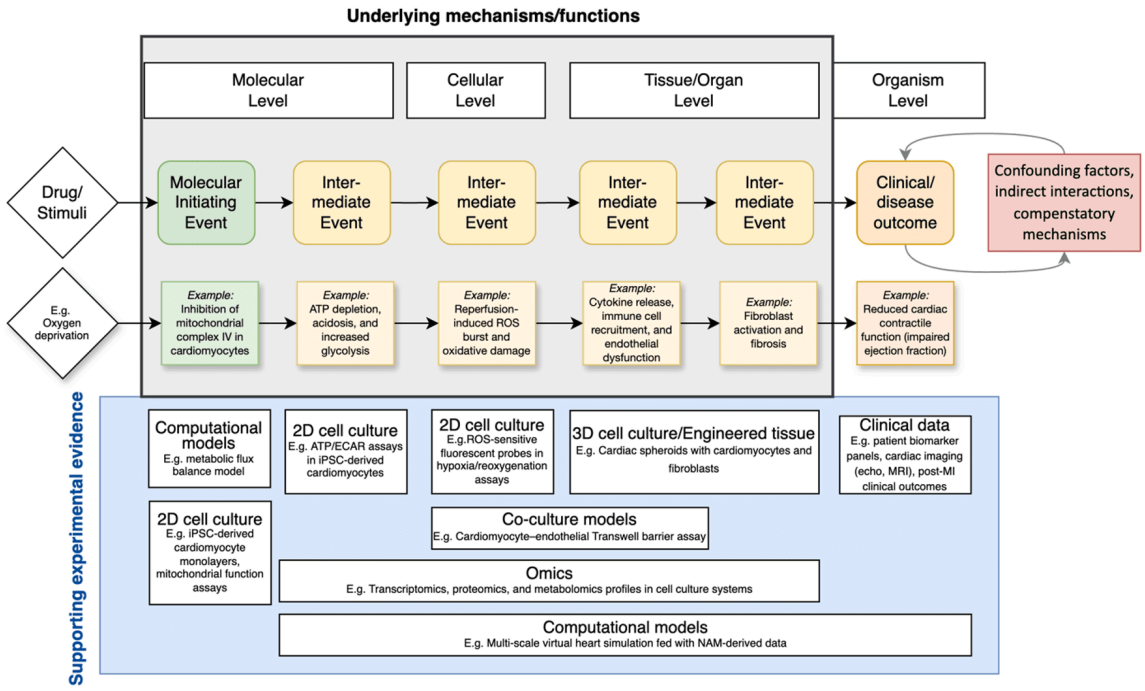

Figure 1. The Clinical Outcome Pathway (COP) framework as a basis to systematically implement NAMs in research questions. NAMs can address specific COP components and be combined to provide a comprehensive view of a research question. Testing strategies can assess multiple COP components in parallel, reflecting the reality of biomedical research where NAMs, clinical studies, and other methods often occur simultaneously.

To illustrate how COPs can operationalize the systematic use of NAMs and help reframe research questions in human-relevant terms, we drafted a prototype COP for myocardial ischemia–reperfusion injury, aligning NAM assays and models with each mechanistic step (Figure 1). By decomposing a disease or therapeutic response into a coherent sequence of causally linked events, from the molecular initiating event to measurable clinical outcomes, COPs specify which biological processes can be interrogated with particular NAM technologies. Locating each NAM within this pathway ensures that experimental data are generated where they are most informative, allows direct comparison with existing *in vivo* evidence, and highlights gaps requiring new model development and acceptance by linking NAM data to clinically meaningful endpoints. Hence, COPs can be used as a translational roadmap to accelerate the development, validation, and implementation of NAMs while preserving continuity with established experimental paradigms.

6. Accelerating the Integration of NAMs into Biomedical Research: A Path Forward

Crucially, using NAMs means creating a deliberate pause between the biological question and the selection of experimental models. A researcher may ask, “How does mutation X influence tumor-cell invasion and metastasis?”, a question that is, by nature, model-agnostic. Before defaulting to a familiar animal study, the question can be broken down into its underlying mechanistic components. Each component is then matched with the most informative NAM or combination of methods and, only where still necessary, a tightly focused *in vivo* experiment. This is not a simple substitution; it is a redesign of the experimental logic. Implementing this shift in addressing research questions requires recognizing the complementary contributions of *in vivo* models and NAMs and fostering constructive dialogue that builds confidence in emerging alternatives.

Initially, NAMs might be positioned as complementary tools, enhancing and supporting animal model-based research methods rather than replacing them outright. This supportive introduction allows for the gradual familiarization of NAMs within the research community. Over time, as the efficacy and applicability of NAMs are rigorously tested and proven across various studies, these methods may play more significant roles within research protocols. This is not about substituting one technique for another but rather comprehensively reassessing how research is conducted, with an emphasis on improving scientific outcomes and ethical standards. As NAMs begin to demonstrate their potential, they can evolve from adjunct tools to integral components of the research process. We envisage that advances in new technologies will enable the scientific questions that are currently answered through animal research to be addressed using NAMs, thereby potentially reducing or even eliminating the need for animal models in certain areas. Ultimately, they may even enable questions that cannot be resolved using animal models to be addressed, such as those related to uniquely human conditions like Alzheimer’s disease. Achieving this goal would not only represent a major scientific and ethical milestone but also align with increasing public pressure to move away from animal testing. This vision for the future, however, requires a foundation built on careful planning, collaboration across disciplines, and a commitment to the continuous development and adaptation of new scientific tools. We thus propose a stepwise approach to enhance the implementation of NAMs in biomedical research:

Step 1: Expanding collaboration between researchers relying on animal models and researchers that develop and use NAMs

To fully exploit NAMs' potential to replace or reduce animal use, fostering collaborations among researchers from different methodological backgrounds is essential (Figure 2). The research community includes those working exclusively with NAMs, those integrating both NAMs and animal models, and those primarily relying on animal models. Researchers using both approaches play a key role in demonstrating how NAMs can complement *in vivo* approaches, identifying where they provide added value, and sharing practical knowledge on their implementation. Their experience can help bridge the gap between NAM developers, engineers or material scientists, clinicians, and researchers focused on biological complexity and disease relevance. For instance, it is often engineers who develop NAMs; although they bring technical expertise, they may lack a focus on biological or disease relevance. On the other hand, clinicians, with their knowledge of clinical presentation and disease relevance in humans, can play a critical role in ensuring NAMs are aligned with clinical realities.

However, each group speaks a different professional "language," and current communication barriers hinder effective collaboration. Thus, promoting interdisciplinary collaboration and mutual understanding is essential. By fostering an environment of active listening, data sharing, and cooperative problem-solving, researchers can ensure that NAMs unite both technical precision and a high degree of biological and clinical relevance.

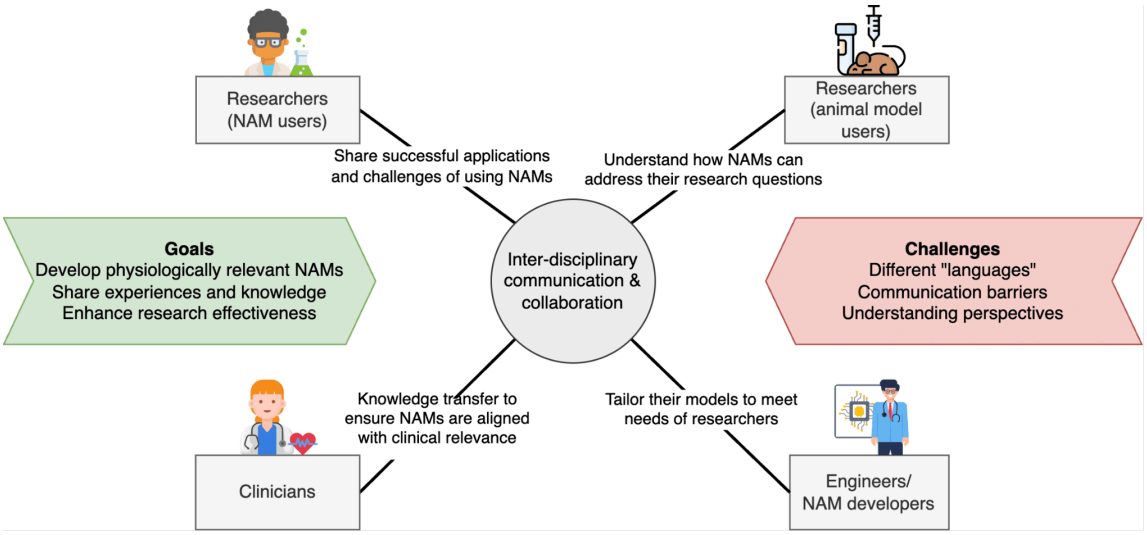

Figure 2. Communication hurdles between researchers, clinicians, and NAM developers are complicating the implementation of NAMs in biomedical research. Interdisciplinary communication and collaboration are needed to reach common goals.

Step 2: Integration through parallel studies

Conducting parallel studies using NAMs alongside animal models can effectively initiate integration. COPs can guide these studies by identifying key biological events where NAMs can produce meaningful, clinically relevant data. Researchers can directly compare outcomes, highlight specific strengths, and determine where and how NAMs are most effective. A critical issue arises when discrepancies occur between NAM and animal model outcomes. In these cases, careful interpretation is needed to discern whether differences stem from true biological species-specific variations, limitations of the NAM model itself, or differences due to experimental conditions. Incorporating intermediate *in vitro* models based on animal cells (e.g., mouse cell-based models alongside mouse studies) may clarify these differences and facilitate translation to human-relevant NAM data. Crucially, however, parallel studies should not lead to additional animal experiments solely to validate NAMs. Ultimately, the benchmark for human-based NAMs should not be animal models but rather the clinical situation or condition they aim to replicate, emphasizing functional and clinically relevant endpoints.

doi.org/10.32388/L13YBA

Step 3: Identifying relevance and evaluating efficacy

To advance beyond mere complementarity, it is crucial to establish the specific contexts in which NAMs can indeed replace and reduce the use of animal models. This requires identifying specific research areas where NAMs can provide equal or superior data compared to animal models and beginning to prioritize these methods for such applications. For this, systematic reviews and meta-analyses can be useful tools to collect comparative data, such as parallel studies, and evaluate the reliability, applicability, and relevance of NAMs in reflecting biological processes and outcomes compared to *in vivo* approaches. Particularly in studies where NAMs have shown the potential to match or exceed the performance of animal models [66][67], NAM use can be expanded to more critical stages of research.

Step 4: Sustained investment and infrastructure development

The transformation of NAMs from complementary tools to primary research methodologies requires not only financial investment but also the development of supportive infrastructure. This includes funding for NAM-focused research projects, incentives for laboratories to acquire and implement advanced NAM technologies, and the creation of shared resource centers that facilitate access to NAMs. Making the use of NAMs more affordable and accessible will accelerate their adoption and integration into mainstream research. Additionally, educational infrastructures must be strengthened to train the next generation of researchers and provide continuous education initiatives for those seeking to engage more in non-animal-based research. A leading example is the newly established Ombion Centre for Animal-Free Biomedical Translation in Utrecht, launched in July 2025 with €124.5 million from the Dutch National Growth Fund [68]. Ombion supports research, education, and the adoption of animal-free methods across various disease areas. Such targeted, large-scale investment makes NAMs more accessible and accelerates their integration into biomedical research.

7. Conclusion

NAMs hold considerable promise for advancing basic biomedical research by providing detailed mechanistic insights and generating data that are directly relevant to human biology. Currently, NAMs can complement in vivo models by enabling high-throughput analyses and offering new perspectives that are often unattainable with animal models alone. In the near future, we envision that NAMs will increasingly surpass animal models given their potential to improve research outcomes by allowing for

more precise experimental manipulation and a deeper understanding of fundamental biological processes and disease mechanisms.

The Clinical Outcome Pathways (COPs) framework can support the systematic integration of NAMs into basic research by mapping how molecular and cellular events relate to broader biological outcomes. This structured approach enables researchers to better align NAM-generated data with relevant research questions, evaluate the strengths and limitations of these methods, and build confidence in their use as reliable complements or alternatives to animal models.

Realizing the full potential of NAMs in basic research will require more than technological advances; it calls for sustained investment in infrastructure, targeted funding for model development, and educational initiatives that equip researchers to design, interpret, and validate experiments based on NAMs. Just as importantly, a shift in scientific mindset is needed: from asking whether an animal experiment is required to considering how a human-relevant, mechanism-driven method might best address the research question. By embracing this approach, the scientific community can establish NAMs as core methodologies in basic biomedical research, driving scientific discovery while advancing the ethical imperative to reduce animal use.

Statements and Declarations

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This article is based upon work from COST Action CA21139, ‘Improving the Quality of Biomedical Science with 3Rs Concepts (IMPROVE),’ supported by COST (European Cooperation in Science and Technology).

References

1. ^*Kaplan BLF, et al. (2024). "Protecting Human and Animal Health: The Road from Animal Models to New Approach Methods." Pharmacol Rev. **76**(1):251–266. doi:10.1124/pharmrev.123.000967.*
2. a, b *ECHA, E.C.A.-. (2023).*

3. ^Schmeisser S, et al. (2023). "New Approach Methodologies in Human Regulatory Toxicology—Not If, But How and When!" Environ Int. **178**:108082. doi:10.1016/j.envint.2023.108082.
4. ^Walder L, et al. (2025). "EU Roadmap for Phasing Out Animal Testing for Chemical Safety Assessments: Recommendations from a Multi-Stakeholder Roundtable." ALTEX. **42**(3):435–450. doi:10.14573/altex.2503241.
5. a, bJRC, J.R.C.-. (2022).
6. a, bLauwereyns J, Bajramovic J, Bert B, Camenzind S, De Kock J, Elezović A, et al. (2024). "Toward a Common Interpretation of the 3Rs Principles in Animal Research." Lab Anim. **53**:347–350. doi:10.1038/s41684-024-01476-2.
7. ^Marshall LJ, Bailey J, Cassotta M, Herrmann K, Pistollato F (2023). "Poor Translatability of Biomedical Research Using Animals—A Narrative Review." Altern Lab Anim. **51**(2):102–135. doi:10.1177/02611929231157756.
8. ^(CAAT), J.H.C.f.A.t.A.T. (1959).
9. ^Kiani AK, et al. (2022). "Ethical Considerations Regarding Animal Experimentation." J Prev Med Hyg. **63**(S2):E255–E266. doi:10.15167/2421-4248/jpmh2022.63.2S3.2768.
10. ^van der Valk J (2022). "Fetal Bovine Serum—A Cell Culture Dilemma." Science. **375**(6577):143–144. doi:10.1126/science.abm1317.
11. ^Pamies D, et al. (2018). "Advanced Good Cell Culture Practice for Human Primary, Stem Cell-Derived and Organoid Models as Well as Microphysiological Systems." ALTEX. **35**(3):353–378. doi:10.14573/altex.1710081.
12. ^Chehelgerdi M, et al. (2023). "Exploring the Promising Potential of Induced Pluripotent Stem Cells in Cancer Research and Therapy." Mol Cancer. **22**:93. doi:10.1186/s12943-023-01873-0.
13. ^Blinova K, et al. (2018). "International Multisite Study of Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes for Drug Proarrhythmic Potential Assessment." Cell Rep. **24**(13):3582–3592. doi:10.1016/j.celrep.2018.08.079.
14. ^Ingber DE (2022). "Human Organs-on-Chips for Disease Modelling, Drug Development and Personalized Medicine." Nat Rev Genet. **23**(8):467–491. doi:10.1038/s41576-022-00466-9.
15. ^Herland A, et al. (2020). "Quantitative Prediction of Human Pharmacokinetic Responses to Drugs Via Fluidically Coupled Vascularized Organ Chips." Nat Biomed Eng. **4**(4):421–436. doi:10.1038/s41551-019-0498-9.
16. ^VPH (2025).
17. ^Nguyen DTT, van Noort D, Jeong I-K, Park S (2017). "Endocrine System on Chip for a Diabetes Treatment Model." Biofabrication. **9**(1):015021. doi:10.1088/1758-5090/aa5cc9.

18. ^Greek R, Kramer LA (2019). "How to Evaluate the Science of Non-Human Animal Use in Biomedical Research and Testing: A Proposed Format for Debate." In: Herrmann K, Jayne K, editors. Animal Experimentation: Working Towards a Paradigm Change. Leiden: Brill. p. 65–87. https://brill.com/display/book/edcoll/9789004391192/BP000003.xml.

19. ^Hall MS, Decker JT, Shea LD (2020). "Towards Systems Tissue Engineering: Elucidating the Dynamics, Spatial Coordination, and Individual Cells Driving Emergent Behaviors." Biomaterials. **255**:120189. doi:10.1016/j.biomaterials.2020.120189.

20. ^Quintard C, et al. (2024). "A Microfluidic Platform Integrating Functional Vascularized Organoids-on-Chip." Nat Commun. **15**:1452. doi:10.1038/s41467-024-45710-4.

21. ^Gray RA, Pathmanathan P (2018). "Patient-Specific Cardiovascular Computational Modeling: Diversity of Personalization and Challenges." J Cardiovasc Transl Res. **11**(2):80–88. doi:10.1007/s12265-018-9792-2.

22. ^Zheng H, Liu X, Liu L, Hu J, Chen X (2025). "Imaging of Endogenous RNA in Live Cells Using Sequence-Activated Fluorescent RNA Probes." Nucleic Acids Res. **53**(2):gkae1209. doi:10.1093/nar/gkae1209.

23. ^Moos F, et al. (2024). "Open-Top Multisample Dual-View Light-Sheet Microscope for Live Imaging of Large Multicellular Systems." Nat Methods. **21**(6):798–803. doi:10.1038/s41592-024-02213-w.

24. ^Luo X, et al. (2025). "Label-Free 3D Photoacoustic Imaging of Tumor Organoids for Volumetric Drug Screening." Adv Sci. :e17226. doi:10.1002/advs.202417226.

25. a, bGarioni M, et al. (2023). "Patient-Derived Organoids Identify Tailored Therapeutic Options and Determinants of Plasticity in Sarcomatoid Urothelial Bladder Cancer." npj Precis Oncol. **7**:112. doi:10.1038/s41698-023-00466-w.

26. a, bFarin HF, et al. (2023). "Colorectal Cancer Organoid-Stroma Biobank Allows Subtype-Specific Assessment of Individualized Therapy Responses." Cancer Discov. **13**(10):2192–2211. PMID 37489084.

27. ^Gardiner LJ, et al. (2020). "Using Human In Vitro Transcriptome Analysis to Build Trustworthy Machine Learning Models for Prediction of Animal Drug Toxicity." Sci Rep. **10**:9522. doi:10.1038/s41598-020-66481-0.

28. ^Kitsara M, et al. (2024). "Introducing the COST Action 'Improving the Quality of Biomedical Science with 3 Rs Concepts' (IMPROVE)." Altern Lab Anim. **52**(6):326–333. doi:10.1177/02611929241286024.

29. ^Cui X, et al. (2024). "Human Midbrain Organoids: A Powerful Tool for Advanced Parkinson's Disease Modeling and Therapy Exploration." npj Parkinson's Dis. **10**:189. doi:10.1038/s41531-024-00799-8.

30. ^Luteijn MJ, et al. (2025). "High-Throughput Screen of 100,000 Small Molecules in C9ORF72 ALS Neurons Identifies Spliceosome Modulators That Mobilize G4C2 Repeat RNA Into Nuclear Export and Repeat-Associated Non-Canonical Translation." Nucleic Acids Res. **53**(7):gkaf253. doi:10.1093/nar/gkaf253.

31. ^Yan Y, et al. (2024). "3D Bioprinting of Human Neural Tissues With Functional Connectivity." Cell Stem Cel l. **31**(2):260–274.e7. doi:10.1016/j.stem.2023.12.009.

32. ^Laverty H, et al. (2011). "How Can We Improve Our Understanding of Cardiovascular Safety Liabilities to D evelop Safer Medicines?" Br J Pharmacol. **163**:675–693. doi:10.1111/j.1476-5381.2011.01255.x.

33. ^Schaffert A, Murugadoss S, Mertens B, Paparella M (2023). "Cardiotoxicity of Chemicals: Current Regulator y Guidelines, Knowledge Gaps, and Needs." ALTEX. **40**:337–340. doi:10.14573/ALTEX.2301121.

34. ^Daley MC, Mende U, Choi BR, McMullen PD, Coulombe KLK (2023). "Beyond Pharmaceuticals: Fit-for-Purp ose New Approach Methodologies for Environmental Cardiotoxicity Testing." ALTEX. **40**(1):103–116. doi:10.1 4573/altex.2109131.

35. ^Conant G, Ahadian S, Zhao Y, Radisic M (2017). "Kinase Inhibitor Screening Using Artificial Neural Networ ks and Engineered Cardiac Biowires." Sci Rep. **7**:11807. doi:10.1038/s41598-017-12048-5.

36. ^Wang Y, et al. (2023). "Emerging Trends in Organ-on-a-Chip Systems for Drug Screening." Acta Pharm Sin B. **13**:2483–2509. https://www.sciencedirect.com/science/article/pii/S2211383523000333.

37. ^Pishas KI, et al. (2024). "High-Throughput Drug Screening Identifies Novel Therapeutics for Low-Grade Se rous Ovarian Carcinoma." Sci Data. **11**:1024. doi:10.1038/s41597-024-03869-x.

38. a, bXu H, et al. (2024). "Tumor-Microenvironment-on-a-Chip: The Construction and Application." Cell Com mun Signal. **22**:515. doi:10.1186/s12964-024-01884-4.

39. ^Gu L, Cai H, Chen L, Gu M, Tchieu J, Guo F (2024). "Functional Neural Networks in Human Brain Organoid s." BME Front. **5**:0065. doi:10.34133/bmef.0065.

40. ^Tal T, et al. (2024). "New Approach Methods to Assess Developmental and Adult Neurotoxicity for Regulat ory Use: A PARC Work Package 5 Project." Front Toxicol. **6**:1359507. doi:10.3389/ftox.2024.1359507.

41. ^Cai Y, Fan K, Lin J, Ma L, Li F (2023). "Advances in BBB on Chip and Application for Studying Reversible Op ening of Blood–Brain Barrier by Sonoporation." Micromachines. **14**(1):112. doi:10.3390/mi14010112.

42. ^Shamul JG, et al. (2025). "Meta-Analysis of the Make-Up and Properties of In Vitro Models of the Healthy a nd Diseased Blood–Brain Barrier." Nat Biomed Eng. **9**:566–598. doi:10.1038/s41551-024-01250-2.

43. ^Schmidt C, et al. (2023). "Multi-Chamber Cardioids Unravel Human Heart Development and Cardiac Defec ts." Cell. **186**(25):5587–5605.e27. doi:10.1016/j.cell.2023.10.030.

44. ^Volmert B, et al. (2023). "A Patterned Human Primitive Heart Organoid Model Generated by Pluripotent St em Cell Self-Organization." Nat Commun. **14**:8245. doi:10.1038/s41467-023-43999-1.

45. ^Dardano M, et al. (2024). "Blood-Generating Heart-Forming Organoids Recapitulate Co-Development of t he Human Haematopoietic System and the Embryonic Heart." Nat Cell Biol. **26**:1984–1996. doi:10.1038/s415

56-024-01526-4.

46. ^Abilez OJ, et al. (2025). "Gastruloids Enable Modeling of the Earliest Stages of Human Cardiac and Hepatic Vascularization." Science. **388**:eadu9375. doi:10.1126/science.adu9375.

47. ^Liu Y, Kamran R, Han X, Wang M, Li Q, Lai D, et al. (2024). "Human Heart-on-a-Chip Microphysiological System Comprising Endothelial Cells, Fibroblasts, and iPSC-Derived Cardiomyocytes." Sci Rep. **14**:18063. doi:10.1038/s41598-024-68275-0.

48. ^Gisone I, Boffito M, Persiani E, Pappalardo R, Ceccherini E, Laurano R, et al. (2025). "Integration of Co-Culture Conditions and 3D Gelatin Methacryloyl Hydrogels to Improve Human-Induced Pluripotent Stem Cells-Derived Cardiomyocytes Maturation." Front Bioeng Biotechnol. **13**:1576824. doi:10.3389/fbioe.2025.1576824.

49. ^Sachs N, et al. (2018). "A Living Biobank of Breast Cancer Organoids Captures Disease Heterogeneity." Cell. **172**(1-2):373–386.e10. doi:10.1016/j.cell.2017.11.010.

50. a, b Tong L, et al. (2024). "Patient-Derived Organoids in Precision Cancer Medicine." Med. **5**:1351–1377. doi:10.1016/j.medj.2024.08.010.

51. ^Gao S, et al. (2021). "CRISPR Screens Identify Cholesterol Biosynthesis as a Therapeutic Target on Stemness and Drug Resistance of Colon Cancer." Oncogene. **40**:6601–6613. doi:10.1038/s41388-021-01882-7.

52. ^Yoon D, Nam Y (2024). "A 3D Neuronal Network Read-Out Interface With High Recording Performance Using a Neuronal Cluster Patterning on a Microelectrode Array." Biosens Bioelectron. **261**:116507. doi:10.1016/j.bios.2024.116507.

53. ^Sato Y, et al. (2022). "Microfluidic Cell Engineering on High-Density Microelectrode Arrays for Assessing Structure-Function Relationships in Living Neuronal Networks." Front Neurosci. **16**:943310. doi:10.3389/fnins.2022.943310.

54. ^Fabbri R, Ahluwalia A, Magliaro C (2023). "Advanced 3D Models for Neuroimmune Interactions: From Organoids to Organ-on-a-Chip Platforms." Cells. **12**(8):1181. doi:10.3390/cells12081181.

55. ^Maurissen TL, et al. (2024). "Modeling Mutation-Specific Arrhythmogenic Phenotypes in Isogenic Human iPSC-Derived Cardiac Tissues." Sci Rep. **14**:2586. doi:10.1038/s41598-024-52871-1.

56. ^Rexius-Hall ML, et al. (2022). "A Myocardial Infarct Border-Zone-on-a-Chip Demonstrates Distinct Regulation of Cardiac Tissue Function by an Oxygen Gradient." Sci Adv. **8**:eabn7097. doi:10.1126/sciadv.abn7097.

57. ^Maringanti R, et al. (2024). "Atherosclerosis on a Chip: A 3-Dimensional Microfluidic Model of Early Arterial Events in Human Plaques." Arterioscler Thromb Vasc Biol. **44**:2453–2472. doi:10.1161/ATVBAHA.124.321332.

58. ^Caballero R, Martinez MA, Peña E (2025). "Fully Coupled Hybrid In-Silico Modeling of Atherosclerosis: A Multi-Scale Framework Integrating CFD, Transport Phenomena and Agent-Based Modeling." Front Bioeng Biotechnol. **13**:1549104. doi:10.3389/fbioe.2025.1549104.

59. ^Flores-Torres S, et al. (2023). "Bioprinted Multicomponent Hydrogel Co-Culture Tumor-Immune Model for Assessing and Simulating Tumor-Infiltrated Lymphocyte Migration and Functional Activation." ACS Appl Mater Interfaces. **15**:33250–33262. doi:10.1021/acsami.3c02995.

60. ^Morrison AI, Sjoerds MJ, Vonk LA, Gibbs S, Koning JJ (2024). "In Vitro Immunity: An Overview of Immunocompetent Organ-on-Chip Models." Front Immunol. **15**:1373186. doi:10.3389/fimmu.2024.1373186.

61. ^EC, E.C.-. (2024).

62. ^Bajard L, et al. (2023). "Application of AOPs to Assist Regulatory Assessment of Chemical Risks: Case Studies, Needs and Recommendations." Environ Res. **217**:114650. doi:10.1016/j.envres.2022.114650.

63. ^a, ^bKorn D, et al. (2022). "Defining Clinical Outcome Pathways." Drug Discov Today. **27**:1671–1678. doi:10.1016/j.drudis.2022.02.008.

64. ^Heesbeen EJ, Bijlsma EY, Risseeuw TA, Hessel EVS, Groenink L (2025). "A Systematic Approach to Identify Gaps in Neuroimmunology: TNF-α and Fear Learning Deficits, a Worked Example." Brain Behav Immun. **123**:752–764. doi:10.1016/j.bbi.2024.10.027.

65. ^Nymark P, et al. (2024). "Building an Adverse Outcome Pathway Network for COVID-19." Front Syst Biol. **4**:1384481. doi:10.3389/fsysb.2024.1384481.

66. ^Passini E, et al. (2017). "Human In Silico Drug Trials Demonstrate Higher Accuracy Than Animal Models in Predicting Clinical Pro-Arrhythmic Cardiotoxicity." Front Physiol. **8**:668. doi:10.3389/fphys.2017.00668.

67. ^Hockney S, et al. (2023). "Next Generation Organoid Engineering to Replace Animals in Cancer Drug Testing." Biochem Pharmacol. **213**:115586. doi:10.1016/j.bcp.2023.115586.

68. ^Utrecht, U. (2025).

Declarations

Funding: This article is based upon work from COST Action CA21139, ‘Improving the Quality of Biomedical Science with 3Rs Concepts (IMPROVE), supported by COST (European Cooperation in Science and Technology).

Potential competing interests: No potential competing interests to declare.